

## Effect of Zinc on Translocation of Iron in Soybean Plants

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### ABSTRACT

Zinc interfered with translocation of iron from roots to above ground parts of *Glycine max.* (L.) Merrill var. Hawkeye. During periods in which zinc impeded iron translocation, it also suppressed the production of reductant by roots. Addition of iron, as a ferric metal chelate (iron ethylenediaminedihydroxyphenylacetic acid), to the growth medium overcame the interference of zinc. In the root epidermis, potassium ferriocyanide formed a precipitate (Prussian blue) with ferrous iron derived from the previously supplied iron ethylenediaminedihydroxyphenylacetic acid. The reduction of ferric iron was suppressed by zinc.

Iron deficiency in plants may be caused by a number of factors that interfere with sorption and translocation of iron. Among these are: microelement imbalance (4), high pH of growth media (10), iron-inefficient plant species (8), low reducing capacity of roots (3), and the oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  (12). Factors employed to correct iron deficiency include genetic variants (5), chelates (9), and the lowering of pH in the growth media (2).

The objective of this study was to characterize the interfering effects of zinc on the translocation of iron. The experimental plant, *Glycine max.* (L.) Merrill var. Hawkeye, which is an iron-inefficient cultivar (13), is well documented relative to the factors associated with iron sorption and translocation.

### MATERIALS AND METHODS

**Nutrient Solution.** The nutrient solution contained, in  $\mu\text{M}$ : Ca, 1250; K, 1000; Mg, 271; N, 3927; P, 65; S, 250; Mn, 27; B, 14; Cu, 0.2; Mo, 0.1; Zn, 0.2; and Fe, 0.4 as  $\text{Fe}^{3+}\text{EDDHA}^1$ . Iron and zinc concentration were varied in some experiments. The initial pH of nutrient solutions was adjusted to 6.0 with 0.1 N NaOH. Nutrient solutions were changed on the 3rd, 7th, and 18th days of growth.

**Plant Growth and Harvest.** Hawkeye soybean seeds were germinated and subsequently grown in aerated nutrient solution in a controlled light chamber under 8-hr dark and 16-hr light periods (1500 ft-c) at  $23 \pm 2^\circ\text{C}$ . Seeds were germinated between layers of moist muslin (prewashed with deionized water) on stainless steel wire frames placed in Pyrex trays. Seeds and trays were covered with aluminum foil for 3 days. Selected 3-day-old seedlings were transferred and grown for 4 days under partial

shading to favor stem elongation. Seven-day-old plants, with stems 10 to 15 cm long, were grouped in two 15-plant bundles per 10-liter Pyrex jar. The nutrient solution volume, unless specified otherwise, was 8 liters. Mild iron deficiency symptoms appeared in the new growth of the plants on the 17th day. Plants were used ("harvested") on the 18th day of growth.

For stem exudate collection, roots were washed briefly with deionized water, and the bundled plant groups were placed individually in 1-liter beakers of aerated nutrient solution. Plant tops were decapitated 10 cm above the roots, and the ends of the cut stems were placed in half-inch Tygon tubing which extended into a calibrated tube in an ice bath. Exudate was collected over a 20-hr period in the dark at  $24^\circ\text{C}$ .

**Cation Isotopes and Assays.** In certain experiments,  $^{59}\text{Fe}$  (7.3 c/g Fe) or  $^{65}\text{Zn}$  (3.94 c/g Zn) was used in nutrient solutions at  $10 \mu\text{C}/\text{l}$  as  $^{59}\text{FeEDDHA}$  and  $^{65}\text{Zn}(\text{NO}_3)_2$ , respectively. Radioassay of  $^{65}\text{Zn}$ , in nutrient solution, was made in a gamma scintillation spectrometer. In stem exudate, total iron and zinc were determined spectrophotometrically by the *o*-phenanthroline method (11), and by atomic absorption analysis, respectively.

Expressed root sap was obtained from 10 g of previously frozen roots. A Carver press was used at  $844 \text{ kg}/\text{cm}^2$  pressure to express the sap.

**Experiments.** Preliminary experiments were done with various concentrations of zinc to determine its effect upon the translocation of iron. From these studies,  $5 \mu\text{M}$  Zn was selected to be used in these studies.

1. *Uptake of Zinc with Time.* Five micromoles of  $^{65}\text{Zn}$  ( $10 \mu\text{C}/\text{l}$ ) were added to the nutrient solution 96 hr before plants were 18 days old. At selected times, 1-ml volumes of the 1-liter nutrient solution were analyzed for  $^{65}\text{Zn}$  and returned to the solution.

2. *Effect of Pretreatment with Zinc.* Five micromoles of zinc were supplied to plants at selected time intervals before stem exudate collection. The effect of zinc, absorbed from the 1-liter nutrient solution before exudate collection of 18-day-old plants, was determined on the subsequent uptake and translocation of  $18 \mu\text{M}$  nutrient iron. In addition, zinc was determined in stem exudate and expressed root sap.

3. *Effect of Iron Concentration on the Inhibitory Effect of Zinc.* The effect of zinc on iron uptake and translocation in 18-day-old plants, decapitated for stem exudate collection, was tested at three iron concentrations: 18, 36, or  $54 \mu\text{M}$   $\text{Fe}^{3+}\text{EDDHA}$ . Zinc was varied from 0.15 to  $15 \mu\text{M}$ .

Additional test plants were used to determine the effect of zinc treatment on the growth of root tips under similar experimental conditions.

4. *Effect of Zinc on the Reducing Capacity of Root Sap and Nutrient Solution.* Zinc was varied from 0.15 to  $15 \mu\text{M}$  in the nutrient solution of 18-day-old intact plants during a 20-hr treatment. Root sap and nutrient solution were assayed for reducing capacity.

The capacity of nutrient solution (50 ml) and expressed root

<sup>1</sup> Abbreviations: FeEDDHA: iron ethylenediaminedihydroxyphenylacetic acid; TPTZ: 2,4,6-tripyridyl-s-triazine.

sap (10 ml diluted to 50 ml with deionized water) to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$ , added as 1  $\mu\text{mole}$  of iron as  $\text{Fe}^{3+}(\text{NO}_3)_3$  in a solution at pH 4.0, was determined spectrophotometrically with 2,4,6-tripyridyl-s-triazine (7). The color of the complex,  $\text{Fe}^{2+}(\text{TPTZ})_2$ , in water conforms to Beer's Law, up to about 60  $\mu\text{M}$   $\text{Fe}^{2+}$ . Formation of the complex is suited to the spectrophotometric determination of  $\text{Fe}^{2+}$  in water from pH 3.4 to 5.8.

**5. Effect of Zinc on Formation of Prussian and Turnbull's Blue in the Root and Nutrient Solution.** An experiment was designed to determine the effect of zinc on the separation of iron from the chelating agent, EDDHA. Potassium ferricyanide was used to demonstrate and to locate iron reduction in the root as iron was released from EDDHA. The internal interaction of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  of ferro-ferricyanide, Turnbull's blue, would produce ferri-ferrocyanide or Prussian blue. Prussian blue, cubic dark blue crystals, would indicate ferrous iron and the area in the root that possessed the greatest reducing capacity.

Roots of intact 18-day-old plants were placed, for 20 hr in the dark, in 1 liter of nutrient solution containing: 10  $\mu\text{C}/\text{liter}$  of  $^{59}\text{Fe}$  as  $^{59}\text{Fe}^{3+}\text{EDDHA}$ ; 18  $\mu\text{M}$   $\text{Fe}$  as  $\text{Fe}^{3+}\text{EDDHA}$ ; 100  $\mu\text{M}$  potassium ferricyanide, and 0.15, 5, or 15  $\mu\text{M}$  zinc as zinc nitrate. After the sorption period, roots were washed with deionized water and examined microscopically for Prussian blue.

In other tests, bundled 18-day-old plants were placed individually in 1-liter beakers of aerated nutrient solution containing zinc, 0.15, 5, or 15  $\mu\text{M}$  as zinc nitrate, and 100  $\mu\text{M}$  potassium ferricyanide or 100  $\mu\text{M}$  potassium ferrocyanide. These tests determined whether either of the cyanide salts, separately in the dark for 20 hr, could form Prussian blue in the absence of the metal chelate,  $\text{FeEDDHA}$ .

Formation of Prussian blue in the dark was also tested in nutrient solution that previously contained plants from the 7th to the 18th day of growth.

Radioautographs were made of selected roots which had been previously pressed between botanical mounting paper and dried in a forced-draft oven at 70 C.

## RESULTS

**Experiment 1. Uptake of Zinc with Time.** The removal of zinc from the growth medium by soybean plants was fairly constant and rapid (Fig. 1A). Figure 1A shows approximately 80% of the zinc removed from the nutrient solution at 48 hr.

**Experiment 2. Effect of Pretreatment with Zinc.** Figure 1B shows that the zinc absorbed, from pretreatment of 5  $\mu\text{moles}$  of zinc at indicated time intervals before exudate collection, interfered with the uptake and translocation of iron. This interference with uptake and translocation of iron was not effective in pretreatments longer than 48-hr.

Stem exudate collections were approximately 15 ml each. Zinc, in expressed root sap, remained near 3  $\mu\text{moles}$  in plants pretreated 24 hr or longer before exudate collection.

Total zinc in stem exudate at 48 hr (Fig. 1B) was approximately 65  $\mu\text{g}$  or 1  $\mu\text{mole}$ . Iron at this time began to increase rapidly in the stem exudate.

**Experiment 3. Effect of Iron Concentration on the Inhibitory Effect of Zinc.** Increasing the iron concentration in the nutrient solution reduced the inhibitory effect of a specific zinc treatment (Table I). Stem exudate volumes (normally 15 ml) were suppressed about 15 to 20% when zinc was 9 or 15  $\mu\text{M}$ , as compared with 0.15 or 4.5  $\mu\text{M}$  zinc.

Additional test plants were used to determine the effect of zinc treatment on the growth of root tips under similar experimental conditions. Zinc suppressed root elongation when supplied at 1.5  $\mu\text{M}$  or higher. However, if, after the 20-hr absorption period in high zinc solution, the plants were placed in a low zinc solution (zinc, 0.2  $\mu\text{M}$ ), root tips as well as root elongation and development appeared normal after 24 hr.

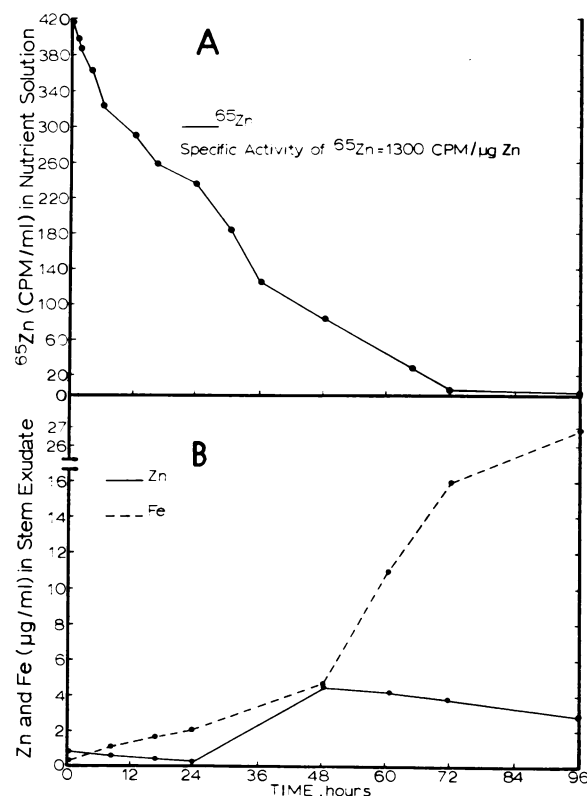


FIG. 1. A:  $^{65}\text{Zn}$  remaining in nutrient solution after addition of  $^{65}\text{Zn}$  96 hr before harvest of soybean plants; B: concentration of Zn and Fe in stem exudate of soybean plants as affected by pretreatment of Zn at indicated time intervals before exudate collection.

Table I. Effect of Various Concentrations of Zn and Fe on the Translocation of Fe by Decapitated Soybean Plants Fe added as  $\text{Fe}^{3+}\text{EDDHA}$ . Sorption period 20 hr.

Level of Zn in Nutrient Solution $\mu\text{M}$	Level of $\text{Fe}^{3+}$ in Nutrient Solution		
	18	36	54
	$\mu\text{moles Fe in total exudate}$		
0.15	11.0	11.6	13.2
0.45	11.8	12.9	13.2
0.90	7.3	10.5	12.6
1.50	4.0	8.9	9.4
3.00	1.9	5.6	7.5
4.50	0.8	3.5	4.6
9.00	0.3	1.3	3.0
15.00	0.2	0.5	1.6

**Experiment 4. Effect of Zinc on the Reducing Capacity of Root Sap and Nutrient Solution.** Increasing the zinc concentration decreased the reduction of iron by expressed root sap and by nutrient solutions (Table II). These results indicate that zinc suppressed the formation of reductant in the roots. The reducing capacity of the nutrient solution measured the reductant released by the roots into the nutrient solution.

**Experiment 5. Effect of Zinc on Formation of Prussian and Turnbull's Blue.** Increasing the zinc concentration decreased the reduction of iron and the accumulation of  $^{59}\text{Fe}$  by the root (Fig. 2). Zinc, 15  $\mu\text{M}$ , suppressed root elongation and formation of Prussian and Turnbull's blue in the root.

Neither potassium ferricyanide nor potassium ferrocyanide

Table II. Effect of Varied Concentrations of Zn, Sorbed over a 20-hr Period, on the Reducing Capacity of the Used Nutrient Solution and of Root Sap of 18-day-old Intact Hawkeye Soybean Plants

Level of Zn in Nutrient Solution	Reducing Capacity ( $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ )	
	Root sap	Nutrient solution
$\mu\text{M}$	$\mu\text{moles}$	
0.15	16	12
0.45	15	9
0.90	14	7
1.50	9	4
4.50	8	2
9.00	4	1
15.00	1	0.2

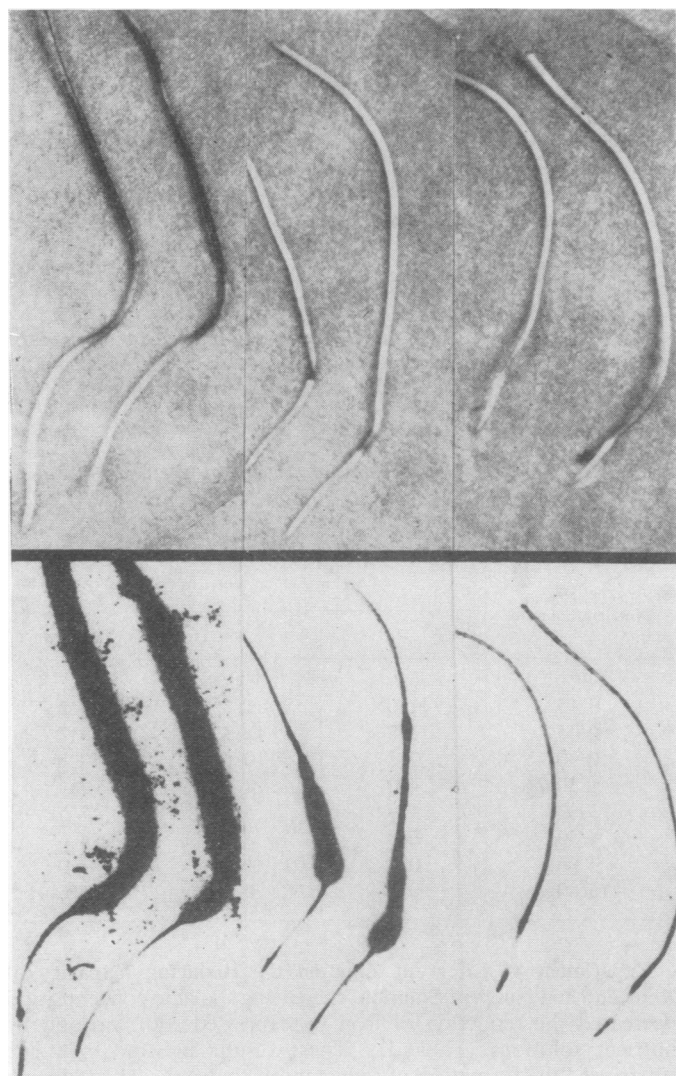


FIG. 2. Effect of 0.15, 4.5, and 15  $\mu\text{M}$  Zn, left to right, on the formation of Prussian blue at the root surface and root elongation. Top row: Photographs; bottom row: radioautographs. (Ferrous iron is required to form the Prussian blue compound.)

formed Prussian or Turnbull's blue in the absence of the metal chelate, FeEDDHA.

Cubic, dark blue crystals (Prussian blue) were found mainly within epidermal walls, and crystals were limited in young

growth to the root tissue in the distal area of the region of root maturation. Turnbull's blue, noncrystalline, occurred with, but to a lesser extent than, Prussian blue.

## DISCUSSION

The uptake of zinc into roots was fairly rapid, although the upward transport of zinc was slow. Plants removed approximately 80% of the original applied zinc (5  $\mu\text{moles}$ ) from the nutrient solution in 48 hr (Fig. 1A). Plants with presorbed zinc (5  $\mu\text{moles}$  in the nutrient solution) for 48 hr had approximately 3  $\mu\text{moles}$  of zinc in the total expressed root sap and 1  $\mu\text{mole}$  of zinc in the total stem exudate. Figure 1 indicates that 1  $\mu\text{mole}$  of zinc in the transport system interfered with translocation of iron at iron concentration of 18  $\mu\text{M}$  as FeEDDHA in the growth medium.

The effect of zinc (Fig. 1) may be extracellular in that change in zinc concentration, after pretreatment with zinc for 24 hr and longer, was mainly in the stem exudate rather than in the root sap. Increase of zinc in stem exudate (Fig. 1B) indicated a decrease of zinc in root tissue. This change paralleled an increase in translocation of iron from the roots. Further increase of iron in stem exudate was associated with a decrease of zinc in the exudate.

Increasing amounts of zinc in the nutrient solution markedly suppressed iron concentration in stem exudate (Table I). However, additional iron concentration in the solution overcame a specific zinc treatment effect. Increase in nutrient iron would be expected to increase the ratio of iron to zinc in the root, thereby reducing the chance of zinc complexing with a system capable of reacting with zinc or iron.

Increase in nutrient zinc concentration also suppressed formation of reductant within the root (Table II) and root elongation (Fig. 2). The amount of reductant within the root and in the growth medium paralleled the decrease of iron in stem exudate (Table I).

Prussian blue, located within epidermal walls, indicated the area of greatest reducing capacity for ferric iron in the root, namely between the regions of root elongation and root maturation. Accumulation of  $^{59}\text{Fe}$  in this specific root area indicated that these cells, at this stage of growth, were the most active in the uptake of iron (Fig. 2). Results, to be discussed in a later publication, indicate this region of the root is more active in the upward translocation of iron than any other region.

Tests *in vitro* in nutrient solution of chlorotic 18-day-old plants presented evidence, during a 20-hr period in the dark at 25 C, pH 4.0, how Prussian blue was formed by the root. First, on the addition of iron, 18  $\mu\text{M}$  as  $\text{Fe}^{3+}(\text{NO}_3)_3$ , and 50  $\mu\text{M}$ , 2,4,6-tripyridyl-s-triazine,  $\text{Fe}^{3+}$  was reduced to  $\text{Fe}^{2+}$  by the root-released reductant and the blue complex,  $\text{Fe}^{2+}(\text{TPTZ})_2$  was formed. Second, iron, 18  $\mu\text{M}$  as  $\text{Fe}^{3+}\text{EDDHA}$  and 100  $\mu\text{M}$  as ferricyanide, was added to similar nutrient solution. Prussian or Turnbull's blue was not found in the nutrient solution. This indicated that, although the reductant could reduce ferric iron when supplied as ferric nitrate, it could not reduce ferric iron when supplied as the metal chelate,  $\text{Fe}^{3+}\text{EDDHA}$ . Therefore, in the formation of Prussian blue in the root, reduction of ferric iron in the metal chelate FeEDDHA appears to occur by the action of factors associated with the reducing system in the root.

In general, for a reaction between a reversible oxidant and reductant system to be practically complete, the potential differences need to be 0.2 to 0.4 v. Normal potentials in aqueous solution, 25 C, are given for the following systems: (a)  $\text{Fe}^{3+} + e \rightleftharpoons \text{Fe}^{2+}$ , 0.78 v; (b)  $\text{Fe}(\text{CN})_6^{3-} + e \rightleftharpoons \text{Fe}(\text{CN})_6^{4-}$ , 0.36 v (6). Accumulation of  $^{59}\text{Fe}$  at the root surface demonstrated that iron was removed from the chelating agent EDDHA and combined with ferricyanide to produce Prussian blue during the 20-hr absorption period (Fig. 2). It is postulated from data given in the formation of Prussian blue, that the redox potential of the

ferro-ferric-EDDHA system is near 0.1 v. The redox potential of various ferro-ferric-chelate systems, for example, ferro-ferric-EDTA, is reported to be near 0.1 v (1).

In soybean, zinc interferes with the translocation of iron by inhibiting the reducing capacity of the root ( $\text{Fe}^{3+} \rightarrow \text{Fe}^{2+}$ ) or accentuates other reactions detrimental to iron transport.

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